CHEMICALS, DRUGS, AND LIPID PEROXIDATION

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INTRODUCTION

Lipid peroxidation is the oxidative deterioration of polyunsaturated lipids. Peroxidative reactions for nonbiological olefinic substances are known. The peroxidative process leads to the formation of free radical intermediates, which can lead to autocatalysis. The effects of free radical formation in radiation-induced reactions are reasonably well recognized. However, the eventual results of free radicals generated during chemically induced lipid peroxidation in vivo are less well understood. Lipid peroxidation in vivo has been said to be of basic importance in aging, in damage to cells by air pollution, in some phases of atherosclerosis, in some forms of liver injury, and in oxygen toxicity (1). However, much of the evidence is indirect and based largely on in vitro findings or the use of antioxidants in vivo. The interpretations made with experiments involving the use of antioxidants have been questioned (2). Nevertheless, lipid peroxidation is a subject of current interest to pharmacologists and toxicologists. The purpose of this review is not to provide an exhaustive treatment of the subject, but to treat sectors of research that are currently receiving considerable attention.

MEASUREMENT OF LIPID PEROXIDATION

The detection and especially the quantitative determination of lipid peroxidation in biological material is not an easy task. Over the last few years, essentially three procedures have gained practical use in measuring lipid peroxidation: the thiobarbituric acid (TBA) reaction, the detection of conjugated dienes, and the measurement of fluorescent products formed by the interaction of peroxidized lipids and other tissue constituents. Among the three methods, the TBA reaction is the most widely used. Since malonaldehyde is a degradation product of peroxidized lipids, the development of a color with the same absorption characteristics (absorption maximum at 532 nm) as a TBA-malonaldehyde chromophore has been taken as an index of lipid peroxidation in a given biological sample. There seems to be general

agreement that the TBA reaction gives a reliable index of lipid peroxidation in tissue extracts. The method has been extensively reviewed and discussed by Barber & Bernheim (3) and Slater (4). Several investigators have attempted to improve the reliability or reproducibility of the test while analyzing biological material (5–10). Failure to detect TBA-reacting material in tissue extracts is not an indication of the absence of lipid peroxidation. Malonaldehyde, if injected, disappears within a few hours from the serum (11) and is metabolized by liver homogenates and fractions thereof (12, 13).

A second method for the detection of lipid peroxidation is to examine tissue extracts for the presence of conjugated dienes by ultraviolet spectrophotometry. The spectra of peroxidized lipids show an absorption at 233 nm, with a shoulder due to ketone dienes between 260 and 280 nm. It is possible, by observing suitable precautions, such as analysis of tissue extracts under strictly anaerobic conditions, to detect the presence of conjugated dienes after treatment in vivo with lipid peroxidizing agents. The methods have been described in detail (14–16).

A comparatively new approach to the measurement of products of lipid peroxidation in tissue is to measure the occurrence of fluorescent products. A variety of molecules that occur commonly in tissue may react with malonaldehyde and yield characteristic fluorescent chromophores (17, 18). Malonaldehyde undergoes decomposition and the decomposition products may also cause production of fluorescent products when they react with proteins (19). Formation of a fluorescent product was observed when DNA was reacted with malonaldehyde (20), or peroxidizing arachidonic acid (21), during peroxidation of phosphatidylethanolamine and phosphatidylcholine and from the reaction of polyunsaturated fatty acids with synthetic phosphatidylethanolamine and phenylalanine (22, 23). Both aqueous and lipid soluble fluorescent products were found in systems that contained peroxidizing polyunsaturated fatty acids and amines; there was good correlation between the formation of fluorescence and oxygen consumption and the formation of TBA reactants. Measurement of these fluorescent products seems to offer a workable way for detecting lipid peroxidation in biological systems and tissues (24–26). Data on the properties and the molecular structures required to produce fluorescence are available (27).

Other methods available for measurement of lipid peroxidation are the iodimetric procedure as described by Bunyan et al (28). An interesting suggestion for measuring lipid peroxidation was made in 1966 by Lieberman & Hochstein (29). In an in vitro system, it was found that the formation of malonaldehyde was paralleled by formation of ethylene, but only when ascorbate and cupric ions were present. Later, it was observed that ethane production was a characteristic of spontaneously peroxidizing mouse tissue, and was found in mice injected with CCl_4 (30). Ethane evolution might therefore be a useful new index of lipid peroxidation in vivo.

LIPID PEROXIDATION AND MICROSOMES

In 1963, Hochstein & Ernster (31) reported that isolated rat liver microsomes formed TBA-reacting material in vitro when they were incubated in the presence

of NADPH and ADP. Antioxidants inhibited its formation. These observations strongly implicated that microsomes would catalyze an ADP-activated peroxidation of lipids, coupled to the NADPH oxidase system (32). The presence of iron in the form of Fe^{2+} seemed essential to activate microsomal lipid peroxidation by ADP and other pyrophosphates (33). The dependence of microsomal lipid peroxidation on the partial oxygen pressure was examined (34). Minimal lipid peroxidation in the presence of NADPH and ADP has a K_m for oxygen of 3×10^{-5}M, i.e. close to the critical oxygen partial pressure of the living cell. CO had no effect on microsomal lipid peroxidation; therefore, it appeared unlikely that cytochrome P450 was involved in the process. The extent of in vitro stimulation of lipid peroxidation depends upon the age and sex of the animal supplying the liver microsomes (35).

The mechanism of in vitro lipid peroxide formation in several tissues was extensively studied (36). Total homogenates from liver, kidney, spleen, heart, or subcellular fractions (nuclear, mitochondrial, microsomal, and soluble) all formed lipid peroxides upon incubation in vitro; they were also active in peroxidizing unsaturated fatty acids. Lipid peroxide formation in microsomes was studied in greater detail (37) and compared to ascorbate-induced lipid peroxidation. Microsomes were fractionated into a supernate, a fluffy layer fraction composed of membranes with a high lipid content, and a sediment. Both NADPH- and ascorbate-induced lipid peroxidation were most pronounced in the fluffy layer fraction. This suggested that the unsaturated fatty acids of the membranes were the substrate for peroxidation in both systems. The role of nonheme iron in lipid peroxidation was more closely analyzed (38). Evidence was found that at least some of the active iron had to be present, as a phosphate complex, and that adenine or adenosine helped to stabilize or orient it in an active configuration. This raised the interesting possibility that iron might be bound to the nucleic acids of the microsomal fraction.

More recent studies have drawn attention to some other features of microsomal lipid peroxidation. Incubation of liver microsomes in the presence of NADPH has led to a loss of cytochrome P450 (39–41). The presence of an antioxidant, butylated hydroxytoluene (BHT), prevented lipid peroxidation and preserved cytochrome P450 (40). Decrease of cytochrome P450 in microsomes under in vitro incubation can be enhanced by CCl_4 and also can be brought about by irradiation of microsomes with ultraviolet light (41). All these changes were parallel to a loss of microsomal polyunsaturated fatty acids and a formation of malonaldehyde. Inhibition of lipid peroxidation by chelating agents, heavy metals, and free-radical trapping agents prevented breakdown of cytochrome P450, and so did some drug substrates; however, other drugs were ineffective in preventing lipid peroxidation (42). Evidence therefore suggests that breakdown of cytochrome P450 is the result of lipid peroxidation and not a prerequisite, although other investigators have reached somewhat different conclusions (43, 44). Electron micrographs of liver and kidney microsomes after in vitro lipid peroxidation are available (45, 46).

Enzymes located within microsomal membranes are affected by lipid peroxidation (46, 47). However, there is no uniform pattern: cytochrome P450 concentration decreases parallel lipid peroxidation. Whereas UDP glucuronyl transferase is activated if lipid peroxidation is moderate, it returns to normal if lipid peroxidation

is extensive. Glucose-6-phosphatase may be affected late during peroxidation, but activity may be preserved in the presence of the substrate, glucose-6-phosphatase.

NADPH–cytochrome *c* reductase, plays an essential role in the peroxidation of microsomal lipids (48). Antibodies prepared against the isolated and partially purified enzyme, which requires EDTA in an optimal concentration for maximum activity, can completely inhibit the NADPH-dependent peroxidation of lipids. Another enzyme involved in the initiation of the reaction is cytochrome b_5 reductase (49). Microsomal lipid peroxidation can be catalyzed not only by NADPH, but as readily by NADH, provided EDTA-Fe is present in the reaction mixture together with ADP-Fe (50). The data suggest that the NADPH-dependent peroxidation of microsomal lipids involved not only NADPH–cytochrome *c* reductase but possibly an additional, not yet identified microsomal electron transport component, which may be replaced by EDTA. On the other hand, in vitro microsomal lipid peroxidation can be inhibited by superoxide dismutase (49, 51). This suggests that superoxide anion and possibly singlet oxygen is instrumental in microsomal lipid peroxidation. Some recent experimental evidence supports this conclusion (52).

It was recognized early that drugs that undergo oxidative demethylation may interfere with and inhibit the NADPH-mediated peroxidation of microsomal lipids. Orrenius et al (53) found that codeine and aminopyrine would strongly prevent the formation of malonaldehyde in rat liver microsomes incubated in the presence of NADPH. Gram & Fouts (54) also observed that aminopyrine, zoxazolamine, aniline, and benzpyrene markedly reduced NADPH-stimulated lipid peroxidation in microsomal membranes. The same authors found that there was a species difference; in rat liver microsomes, considerably more TBA-reacting material was found than in rabbit liver microsomes. Wills (55) suggested that the system involved in lipid peroxidation was at least partially identical with the drug-hydroxylating system. He found a close parallelism between formation of malonaldehyde and loss of activity of certain microsomal enzymes. The following enzymes were sensitive to lipid peroxidation: glucose-6-phosphatase, oxidative demethylation of aminopyrine, and *p*-chloromethylaniline, hydroxylation of aniline, NADPH-oxidation, and menadione-dependent NADPH-oxidation; on the other hand, NAD-NADP glycohydrolase, ATPase, esterase, and NADPH-cytochrome *c* reductase were insensitive or only slightly inhibited (47). Therefore, certain substrates may actually protect microsomes against lipid peroxidation. A recent, interesting example is the potent carcinogen aflatoxin B_1; oxidative metabolism of aflatoxin B_1 retards lipid peroxidation (56).

Induction of lipid peroxidation with Fe^{2+} in isolated microsomes leads to an immediate sharp decline of ethylmorphine demethylase activity (57), and preincubation of microsomes with ascorbic acid or a NADPH-generating system reduces aminopyrine, ethylmorphine, and codeine demethylase (58). Contrary to other observations (39–41) the content of the microsomes in cytochrome P450 and the activities of NADPH–cytochrome *c* reductase and NADPH–neotetrazolium diaphorase apparently were only slightly affected by the stimulation of lipid peroxidation in these experiments. Treatment of the microsomes with CO inhibited

aminopyrine N-demethylation, but increased lipid peroxidation; this observation seems to contradict the earlier findings by Hrycay & O'Brien (43, 44) that cytochrome P450 would be instrumental in inducing lipid peroxidation.

More recently, Jacobson et al (59) examined the kinetics of acetanilide hydroxylation and pentobarbital oxidation by rat liver microsomes and found that the deviation from linearity for product formation was attributable at least in part, to lipid peroxidation and the associated breakdown of cytochrome P450, an idea originally put forth by Gram & Fouts (54). They also confirmed that rat liver microsomes underwent rapid lipid peroxidation in vitro, whereas in microsomes from rabbit liver, practically no malonaldehyde formation occurred and the loss of cytochrome P450 was less than 10%. Similar species differences were also observed by Kamataki & Kitagawa (60); in rat liver microsomes, lipid peroxidation was most extensive; then came guinea pigs, mice, and rabbits. There was no good correlation between the extent of lipid peroxidation and the loss of microsomal activity.

LIPID PEROXIDATION AND LUNG INJURY

During the last few years, considerable attention has been focused on the pathological and biochemical changes brought about in lung tissue by oxygen and oxidant gases nitrogen dioxide and ozone. Nitrogen dioxide and ozone are essential ingredients in the so-called photochemical smog. Pulmonary oxygen toxicity may be a serious complication in the prolonged treatment of certain cardiorespiratory diseases and may also be a problem in aerospace technology. There are several recent reviews on the mechanism and the anatomo-pathological and functional consequences of oxygen toxicity in the lung (61–63), and it is generally thought that lipid peroxidation may be an important consequence of exposure to normobaric or hyperbaric oxygen. Lipid peroxides are readily formed during incubation of homogenates from many tissues under oxygen (36). In lung homogenates obtained from α-tocopherol-deficient animals, significantly greater lipid peroxidation occurred during a 2-hr incubation period than did in lung homogenates obtained from normal animals (64). However, prior to the incubation, identical amounts of TBA-reacting material were found in the lungs from the deficient animals compared to nondeficient animals. Exposure of both normal and α-tocopherol-deficient animals to hyperbaric oxygen failed to raise the concentrations of lipid peroxides in the lung in vivo, although formation was enhanced in deficient lungs upon in vitro incubation. However, if homogenates of normal animals exposed to hyperbaric oxygen were incubated in vitro, lipid peroxidation was inhibited. No inhibition was observed if Fe^{2+} and ascorbic acid were added to the homogenates prior to the incubation; lipid peroxidation proceeded at a normal rate. The possibility was raised that in vivo hyperbaric oxygen would oxidize ascorbic acid and Fe^{2+}, leaving the lungs too depleted of these elements, thus permitting the occurrence of in vitro lipid peroxidation.

Direct evidence for the in vivo production of lipid peroxides in lung has been obtained from experiments with nitrogen dioxide and ozone. In vitro experiments

established that both gases are capable of oxidizing fatty acid methyl esters (65). The lungs of rats exposed to NO_2 (1 ppm for 4 hr) were analyzed for peroxidized polyenoic fatty acids (66). Difference spectra were obtained which were characteristic of diene conjugation. The changes reached a maximum between 24 and 48 hr after exposure. Pretreatment of the animals with large doses of α-tocopherol partially prevented the development of lipid peroxidation. Ultraviolet absorption spectra characteristic of diene conjugation were also found in the lipids extracted from the lungs of mice exposed to ozone (0.4–0.7 ppm for 4 hr) (67). It was pointed out that the levels of both the ozone (67) and the nitrogen dioxide (66) to which the animals were exposed were not above concentrations to which populations in major urban centers may frequently be exposed. However, in vitro exposure of erythrocytes to ozone is also a procedure that enhances formation of lipid peroxides, as measured by the TBA reaction (68). It is conceivable that other oxidant gases will have similar effects. Since exposure to ozone may cause considerable blood stasis within the pulmonary vasculature, it has to be recognized that at least part of the observed lipid peroxidation might not actually occur in the lung cells themselves, but could be due to an increased presence of red blood cells.

In another series of experiments, it was observed that animals exposed to various concentrations of ozone had substantially more TBA-reacting material in their lung homogenates than had animals not exposed to ozone. Supplementing the diet with α-tocopherol decreased, but did not completely abolish the observed increase in lipid peroxidation (69, 70). In the same experiments, it was found that although the TBA reaction detected the formation of lipid peroxides, analysis of lung tissue with the fluorescent techniques developed by Tappel and his associates (17–26) gave negative results. Mustafa et al (71) examined the respiration of lung homogenates and isolated mitochondria from the lungs of ozone-exposed rats, and also exposed mitochondria in vitro to ozone. Acute in vivo exposure or in vitro exposure decreased the respiratory rate of mitochondria; increased production of TBA-reacting material and evidence for diene conjugation was observed if mitochondria were exposed to ozone in vitro. However, in homogenates of lungs from animals exposed to ozone in vivo, no increased amount of lipid peroxidation could be detected. This made it questionable whether the decreased rate of mitochondrial respiration was indeed due to lipid peroxidation in the mitochondrial membranes. Later work (72) confirmed that in vitro exposure of lung mitochondrial suspension to ozone results in a 5- to 6-fold greater production of TBA-reacting material, whereas no such evidence was found in the homogenates subsequent to in vivo ozone exposure. Presence of vitamin E did not protect against in vitro lipid peroxidation by ozone, but the presence of ascorbate and glutathione greatly enhanced it. Indication for in vivo peroxidation of lung lipids, as measured by increased ultraviolet absorption at 235 nm, was also observed in the lungs of rabbits exposed to 1 ppm of ozone for 4 hr (73). Indexes of erythrocyte lysis and membrane damage were not observed under these circumstances, and the ozone lesion seemed to be confined to lung tissue only. Exposure of rabbits to 1 ppm of ozone for 90 min produced an appreciable decrease in lung microsomal cytochrome P450 content, which was evident immediately following ozone exposure and persisting for about 5 days. In vitro studies

confirmed the in vivo results inasmuch as ozone produced a loss of cytochrome P450, together with the production of TBA-reacting material (74).

Studies on the effects of oxidant gases upon lung biochemistry now begin to show that the lung has an effective mechanism with which it may protect itself against oxidant-induced lipid peroxidation (75–78). The most prominent features are increases in the activities of the enzymes 6-phosphogluconate dehydrogenase (6PGDH), glucose-6-phosphate dehydrogenase (G6PDH), glutathione reductase, and glutathione peroxidase. Nitrogen dioxide has essentially similar effects, as did ozone, although it changed glutathione peroxidase activity only insignificantly. Increased activities of the same enzymes were also observed during exposure to oxygen (77). It is possible that the observed changes in enzyme activity represent a protective mechanism. Increased activity of G6PDH and of 6PGDH most probably reflects an increased activity of the pentose pathway. One consequence of this might be to maintain adequate concentrations of cellular NADPH and glutathione. Thus the cells would be protected against lipid peroxidation. The view that increased production of NADPH seems to be vital to protect lung cells of ozone-exposed animals also comes from the observation that the addition of α-tocopherol to the diet practically prevents an increase in the NADPH-generating enzyme system, but had practically no effect on the activity of other enzymes involved in glucose metabolism. However, increases in the enzymes involved in the pentose pathway and the maintenance of adequate glutathione supply are probably not the only protective mechanisms the lung disposes of for protecting itself against oxidant-inflicted lipid peroxidation. Crapo & Tierney (79) found recently that tolerance to oxygen is accompanied by an increase in pulmonary superoxide dismutase. This enzyme transforms the free radical superoxide anion to a less toxic form and, by doing so, might prevent the formation of lipid peroxides.

Lipid peroxidation in lung need not necessarily be produced by oxidant gases only. The herbicide paraquat, if ingested or injected parenterally, will produce extensive lung damage (80). The lesion is characterized by acute edema, death of alveolar epithelial cells and, in later stages, by a progressive interstitial fibrosis of the lung. In rats, paraquat toxicity is greatly enhanced by oxygen (81). This prompted Bus et al (82) to examine whether the toxicity of paraquat might be due to lipid peroxidation. Incubation of paraquat with liver microsomes or a system containing NADPH-cytochrome *c* reductase, NADPH, and microsomal lipid greatly increased the formation of malonaldehyde; the amount of malonaldehyde formed was dependent upon the concentration of paraquat in the incubation mixture. Jlett et al (83), on the other hand, found that paraquat inhibited in vitro lipid peroxidation. However, in more recent experiments, Bus et al (84) were able to provide additional evidence that paraquat might, in vivo, peroxidize pulmonary lipids. Paraquat toxicity was significantly enhanced in selenium- or vitamin E–deficient mice or mice pretreated with diethyl maleate. Other evidence that paraquat may exert its toxic action through formation of singlet oxygen and subsequent lipid peroxidation has been provided (85); rats can be partially protected against paraquat toxicity by petreatment with the enzyme superoxide dismutase. Lipid peroxidation in lung may therefore occur also by drugs that reach the lung via the bloodstream.

LIPID PEROXIDATION AND LIVER INJURY

Injury Resulting in Necrosis

Since 1965 there has been considerable work by several investigators that indicates that lipid peroxidation could be one of the principal causes for the hepatotoxicity produced by CCl_4. Earlier investigators had demonstrated that the liver injury produced by CCl_4 could be prevented or greatly modified by pretreating animals with various antioxidants. This work was reviewed in detail by Recknagel (86). In vitro CCl_4 could accelerate the lipid peroxidation of rat liver homogenate (87, 88) and exhibited a prooxidant effect on rat liver microsomes (87, 89); this increase in lipid peroxidation was associated with a loss in glucose-6-phosphatase activity. Subsequently it was shown that in vivo lipid peroxidation also occurred shortly after the administration of CCl_4 to rats. These data have been reviewed in detail by Slater (4) and by Recknagel & Glende (90). It is now reasonably established that CCl_4 is activated to a free radical in vivo, that lipid peroxidation occurs very quickly in microsomes prepared from damaged livers, that this lipid peroxidation occurs within minutes after the administration of CCl_4 in vivo, that the lipid peroxidation is associated with loss of enzyme activity of microsomes prepared from livers, and that various antioxidants or free radical-trapping agents can protect animals against the hepatotoxic effects of CCl_4. Slater & Sawyer (91) compared the prooxidant effect of CCl_4 to those obtained with $CBrCl_3$, $CFCl_3$, and $CHCl_3$. They found that $CBrCl_3$ was much more potent as a prooxidant than CCl_4. $CFCl_3$ although much weaker than CCl_4 was more potent than $CHCl_3$. The relative order of activity in this in vitro system was what would be expected if homolytic bond fission of the halomethane had stimulated lipid peroxidation. CHI_3 has been found to produce liver injury which is quite similar to that produced by CCl_4; with this substance it has also been possible to demonstrate the lipid peroxidation in vivo by the formation of conjugated dienes (92).

Although it seems reasonably established that lipid peroxidation does occur with CCl_4-induced liver injury, there is still some controversy regarding the relative importance of this effect in the subsequent pathologic changes. Some of the various points of controversy are contained in the reviews by Slater (4) and by Recknagel & Glende (90). Recknagel and his co-workers, who have been the chief proponents of the lipid peroxidation hypothesis, do recognize that there are a number of unknown factors that can contribute to cell death after lipid peroxidation has occurred. They state that there is not a one-to-one correspondence between the initial lipid peroxidation and the eventual development of necrosis. Unfortunately in a number of experiments that have been used to unravel the relative importance of lipid peroxidation, death of the animal has been used as the biological end point. As Slater (4) points out, the cause of death in acute CCl_4 intoxication is not known; CCl_4 can affect the central nervous system, the cardiovascular system, and kidney function. Therefore, conclusions drawn on survival of animals can be precarious unless the actual cause of death is established.

Comporti et al (93) have reported that N,N'-diphenyl-*p*-phenylenediamine (DPPD), an antioxidant that protects against CCl_4-induced necrosis, did not pre-

vent the production of conjugated dienes in microsomal lipids in vivo. Cystamine, cysteine, and pyrazole, which decrease the binding of CCl_4 to cellular components, do not change the extent of lipid peroxidation, although necrosis is partially prevented (94–97). Recently Díaz Gómez et al (98) have studied species differences in CCl_4-induced hepatotoxicity. They found that the degree of necrosis decreased in the following order: mouse, guinea pig, hamster, rat. The chicken was resistant to CCl_4. The degree of irreversible binding of CCl_4 to cellular components decreased in the same order. However, the intensity of CCl_4-induced lipid peroxidation decreased in a different order: rat, hamster, guinea pig, chicken, mouse. There was a better correlation between the intensity of the process of irreversible binding of CCl_4 to cellular components and necrosis than there was between lipid peroxidation and necrosis. In the mouse, the necrotic process was seen in the absence of lipid peroxidation during the 3- to 24-hour period of observation. These authors could find no evidence of lipid peroxidation in mice.

Recently, the effects of vitamin E on CCl_4-induced lipid peroxidation and the increase in liver triglycerides have been studied (99). With a large dose of CCl_4, there was a good correlation between the amount of lipid peroxidation in the liver and the degree of triglyceride accumulation; vitamin E treatment could partially reduce lipid peroxidation and also reduced steatosis in rats. However with low doses of CCl_4 there was no correlation between these events; administration of vitamin E did not affect the incorporation of CCl_4 into microsomal lipids nor did it affect the reduction in glucose-6-phosphatase activity induced by CCl_4. de Ferreyra et al (100) studied the protective effects of a number of antioxidants (DPPD,α-tocopherol, promethazine). At doses that protected against CCl_4, these antioxidants had no effect on the production of conjugated dienes in microsomal lipids after the administration of CCl_4. Therefore, caution is urged in the use of antioxidant protection as supporting evidence for lipid peroxidation.

Torrielli et al (101), on the other hand, were able to show that the antioxidant DPPD could reduce the amount of lipid peroxidation which occurs in liver microsomes after intoxicating doses of CCl_4. The amount of conjugated dienes were markedly reduced 30 min after the administration of the hepatotoxin; they also showed that recovery from the peroxidative alterations seemed to occur more quickly in DPPD-protected rats. The metabolic activation of CCl_4, however, was unaffected by pretreatment with DPPD. They found that DPPD could also act as an inhibitor of enzymes bound to the endoplasmic reticulum and that this substance has properties other than antioxidant ones.

Selenium can reduce significantly the amount of malonaldehyde present in the livers of animals treated with CCl_4 (102). This is consistent with the known antioxidant properties of selenium. However, selenium treatment apparently has no effect on the formation of conjugated dienes 2 hr after administration of CCl_4. The protective effects of selenium have been attributed to enhanced activity of glutathione peroxidase, which brings about the destruction of excess lipid peroxides by reducing them to hydroxy acids, thus diminishing the formation of malonaldehyde; the lack of effect of selenium on conjugated dienes was attributed to the enhanced formation of hydroxy acids. Benedetti et al (102) found that CCl_4 did not affect glutathione peroxidase activity.

When the lipid peroxidation hypothesis was introduced it appeared that a universal mechanism for the production of liver injury might have been uncovered. It was expected that the halogenated hydrocarbons related to CCl_4, which also produce liver necrosis, would also exhibit prooxidant activity. With $CHCl_3$ this has not been found to occur. In vitro $CHCl_3$ does not exert a prooxidant effect on the liver microsomal lipids (90, 103, 104). Klaassen & Plaa (104) attempted to find evidence for the formation of conjugated dienes in microsomes from animals treated with $CHCl_3$; in parallel studies they were able to demonstrate conjugated dienes following CCl_4. However they could never find increased conjugated dienes after $CHCl_3$ administration, regardless of the time period studied. These authors and others (104–106) also failed to detect a decrease in hepatic glucose-6-phosphatase activity after the administration of $CHCl_3$ in vivo.

Recently it has been reported that rats pretreated with phenobarbital will produce conjugated dienes in hepatic microsomal lipids after 2 hr of $CHCl_3$ anesthesia (103, 107); however, rats not treated with phenobarbital did not show this response to $CHCl_3$ anesthesia. Lavigne & Marchand (108) reported that hepatic glucose-6-phosphatase activity was depressed in phenobarbital-pretreated rats given $CHCl_3$ but that this response was absent in rats not treated with phenobarbital.

These data have been difficult to reconcile with the lipid peroxidation theory because $CHCl_3$ produces the same extensive liver injury seen after CCl_4. Slater (4) has argued that the amount of conjugated dienes formed following $CHCl_3$ administration might occur at a rate considerably slower than that produced by CCl_4 and well within the limits of the biotransformation capacity of the hepatocyte. He proposed that the lack of evidence for conjugated dienes in vivo may merely indicate that the products of lipid peroxidation are metabolized too rapidly to permit accumulation and detection by the present analytical tests. Klaassen & Plaa (104) have also pointed out the possibility that the techniques devised for the determination of conjugated dienes in vivo might be too insensitive to detect that degree of lipid peroxidation necessary to produce liver injury. In effect, the possibility occurs that the conjugated dienes that are measured after CCl_4 administration represent an amount of lipid peroxidation that is much greater than that necessary to produce the degenerative changes. Therefore, the question whether $CHCl_3$ produces lipid peroxidation or not depends upon the devising of more sensitive analytical techniques for the detection of lipid peroxidation in vivo. In this regard it should also be noted that even with CCl_4 the cells responsible for the production of conjugated dienes in vivo have not been identified histochemically. The cell type and their location in the hepatic lobule are yet to be determined.

Recently the effects of 1,1-dichloroethylene have been studied in rats (109). This chlorinated hydrocarbon causes liver injury that is quite comparable to that produced by CCl_4. It was found that 1,1-dichloroethylene causes reduction of hepatic glucose-6-phosphatase activity, causes an increase in hepatic triglycerides, and appears to inhibit microsomal mixed function oxidases; these effects are all similar to those of CCl_4. 1,1-Dichloroethylene does not produce lipid peroxidation in vitro or in vivo; conjugated dienes were not increased over a 20-hr span after the administration of the substance.

Ethylene dibromide can produce liver injury in rats; however, the accumulation of triglycerides is considerably less than that obtained with CCl_4 (110); this substance also produces the appearance of conjugated dienes in rat liver microsomal lipids 2 hr after its administration in vivo. However, this compound does not produce a prooxidant effect on microsomal lipids in vitro (111); it was concluded that the lipid peroxidation after ethylene dibromide did not seem to be directly related to its toxicity. Dimethylnitrosamine, which also produces liver injury, has been shown to be a prooxidant of rat liver microsomes in vitro (112) but does not cause production of conjugated dienes in vivo. Thioacetamide has also been found not to produce enhanced lipid peroxidation in hepatic microsomes in doses that produced liver injury (113). Brown et al (103, 107) have studied the effects of halothane on conjugated diene contents of rat liver following anesthesia by inhalation. Two hours after anesthesia no increase in conjugated dienes was observed in the microsomal lipids in any of the animals. However, when the rats were subjected to phenobarbital treatment for 5 days prior to the anesthesia, halothane was found to cause noticeable increases in conjugated dienes. The maximum increase in conjugated dienes occurred 1 hr after anesthesia, and the concentration was back to normal by 16 hr. Halothane, when added to incubations of hepatic microsomes, did not cause an increase in TBA reactants in vitro.

Injury Resulting in Lipid Accumulation

In 1964, DiLuzio (114) reported that ethanol-induced fatty liver could be modified by treating animals with antioxidants. This led to the formulation of the hypothesis that lipid peroxidation was an important factor in the production of fatty liver caused by ethanol. The subject is still rather controversial. Hashimoto & Recknagel (115) carried out studies to show whether there was any direct evidence for the formation of lipid peroxides; they concluded that there was no chemical evidence for hepatic lipid peroxidation in acute ethanol toxicity. However, DiLuzio (116, 117) has summarized evidence that is consistent with the idea that enhanced lipid peroxidation occurs with acute ethanol intoxication. Rats pretreated with ethanol exhibited an increased amount of peroxides in lipids (118); the peroxides were measured by an iodimetric method using purified lipid extracts. The applicability of this technique for measuring lipid peroxides in tissues has been questioned (4, 115). The intravenous administration of coenzyme Q_4 was shown (118) to abolish the elevation in liver triglycerides and also diminish the peroxide content of the liver lipid. It was later demonstrated (119) that various other antioxidants could protect animals against the ethanol-induced fatty liver. Subsequently, lipid peroxidation, as estimated by the TBA reaction in liver homogenates obtained from animals treated with ethanol, was shown to be significantly increased (120). Enhanced TBA reactants could be detected for 6 and 12 hr after the oral administration of ethanol; the greatest concentration was observed in the 12-hr ethanol-treated group. In vitro the addition of ethanol to rat liver homogenates resulted in enhanced lipid peroxidation. The suitability of the TBA reaction for measuring in vivo lipid peroxidation has also been questioned (115). While Recknagel's group has been unable to find increased formation of conjugated dienes in microsomal and mitochondrial liver lipids follow-

ing ethanol administration, DiLuzio has reported (116, 117) that ethanol administration results in an increase in conjugated dienes in liver mitochondria but not in liver microsomal lipids; these increases were observed 1 hr after the administration of ethanol in vivo. In the same experiments DiLuzio was able to show that CCl_4 caused increases in conjugated dienes only in microsomal lipids but not in mitochondrial lipids, thus confirming Recknagel's observations. Scheig & Klatskin (121) were also unable to show increased formation of malonaldehyde in homogenates of rat liver containing cell sap and microsomes but no mitochondria, following treatment with ethanol.

More recently Comporti et al (122) were able to show that liver homogenates from animals treated with ethanol exhibited increased malonaldehyde formation. The increase in activity seemed to be due to a component present in the soluble supernatant fraction of the homogenates rather than one that was from the mitochondria or from the microsomes. MacDonald (123) measured conjugated dienes in mitochondrial and microsomal lipids from livers of rats 3 hr after treatment with ethanol. This author confirmed the finding that mitochondrial lipids showed enhanced conjugated diene formation whereas microsomal lipids did not; however, lipid peroxidation was found to occur in only one half of the animals treated with ethanol. The other animals showed no evidence of conjugated dienes; the reason for this variation is unknown.

Evidence for enhanced formation of lipid peroxides has been obtained in rats fed alcohol for 1 month (124). In these experiments microsomes obtained from livers were found to form more malonaldehyde in vitro than control livers. A decrease in polyenoic fatty acids in the microsomes of the ethanol-treated animals was observed, which would be consistent with a peroxidative destruction of membrane lipids.

DiLuzio & Hartman (125) have postulated that ethanol causes lipid peroxidation by reducing the endogenous antioxidant activity of the tissue. They envisaged a balance between autoxidation and antioxidant activity occurring in normal tissue. They postulated that ethanol administration would result in a decrease in endogenous antioxidant activity and that this would be found particularly in the mitochondria of ethanol-treated livers. Experimentally this seems to have been confirmed; ethanol administration produced a significant reduction in endogenous lipid-soluble antioxidant content of hepatic mitochondria, but not in microsomes. In the same experiments, CCl_4 resulted in a significant reduction in lipid-soluble antioxidant activity in microsomes but not in the mitochondrial fraction. They suggested that the losses observed in the antioxidant content of the subcellular particles were directly attributable to a free radical attack on the lipoprotein membranes of the respective subcellular components.

Orotic acid when added to the diet of rats will cause fatty livers. The addition of 1.5% orotic acid to the diet will produce a three-fold increase in liver lipids within 4 days. This increase in liver lipids is prevented by pretreating animals with DPPD (126). Liver lipids obtained from animals fed orotic acids will undergo lipid peroxidation, as measured by the TBA reaction, much more rapidly in vitro than liver lipids from control animals (127, 128). The composition of the lipids obtained from

these animals was unchanged; in particular no reduction in polyunsaturated fats was observed (128). Torrielli et al (129) demonstrated that the pretreatment of animals with DPPD prevented the enhanced formation of lipid peroxides. However, these investigators also searched for the presence of conjugated dienes in microsomal lipids from animals treated with orotic acid; 2–8 days after the dietary administration of orotic acid, it was not possible to detect the enhanced formation of conjugated dienes. They, therefore, concluded that orotic acid does not function like CCl_4 in that conjugated dienes are not formed more rapidly in animals fed orotic acid. They proposed that perhaps orotic acid interferes with endogenous antioxidants present in the liver and thereby alters the rate of the TBA reaction in vitro. They also proposed that the protective effect of DPPD on hepatic triglyceride accumulation did not necessarily indicate that peroxidation was the initiating event in this response. They proposed that DPPD could be protecting by some other mechanisms, because they had demonstrated that this antioxidant is also capable of decreasing enzyme activity in the endoplasmic reticulum. While it now appears that animals fed orotic acid do seem to have some enhanced formation of lipid peroxide, this process does not seem to be the major reason for the initiation of fatty livers.

Peroxidative decomposition of microsomal lipids has also been demonstrated to occur in rats given yellow phosphorus (130). In these experiments, conjugated dienes were found to be elevated in microsomal lipids obtained from animals 4 hr after the administration of phosphorus. At this time liver lipids had not increased significantly. It was also found that the vesiculation of the endoplasmic reticulum and ribosomal dispersion was prominent 12 hr after the administration of phosphorus. Mitochondria were still normal at this time. In this situation, therefore, it appears that lipid peroxidation could be a factor in the fatty liver induced by phosphorus because the appearance of conjugated dienes occurred rapidly, preceded the changes in hepatic ultrastructure, and preceded the increase in liver lipids. In this regard the sequence of events with phosphorus is qualitatively similar to that observed after CCl_4. DiLuzio (131) has shown that an antioxidant can protect animals against the effect of phosphorus.

There has been some interest in determining whether the fatty liver caused by choline-deficient diets can also be explained by enhanced lipid peroxidation. Ghoshal et al (132) measured the presence of diene conjugation in microsomal liver lipids and in mitochondria of rats 3 to 12 hr after the administration of a choline-deficient diet. They also measured conjugated dienes in the fraction of weanling rats 5 days after the commencement of the choline-deficient diet. The concentration of hepatic triglycerides progressively increased in these animals and by the twelfth hour was significantly higher than those obtained in controls. However, there was no evidence in microsomes or in mitochondria that enhanced formation of conjugated dienes had occurred. There was an absence of enhanced lipid peroxidation in the livers of weanling rats fed a choline-deficient diet for 5 days; these authors therefore concluded that lipid peroxidation is not a factor in the development of hepatic changes associated with early choline deficiency.

CONCLUSION

In this article, we review some experiments, all of which were designed to shed some light on the question whether peroxidation of structural and functional lipids by drugs and chemicals can cause cell and tissue damage. A sound and critical appraisal of the significance and implications of this concept seems more than overdue. However, the data reviewed, as well as many others omitted for lack of space, do not yet seem to allow this.

Peroxidation of unsaturated lipids by molecular oxygen is a straightforward chemical process, well understood and well analyzed. In artificial or natural membrane systems, chemicals may readily produce changes associated with lipid peroxidation. Under appropriate in vitro conditions, microsomal membranes in particular, but also mitochondrial and lysosomal membranes, do show loss of functional integrity when lipid peroxidation appears to occur. However, the seemingly most crucial piece of evidence, the presence of peroxidized lipids in vivo, is more often lacking than not. This, of course, does not mean that lipid peroxidation does not occur in vivo. Absence of evidence is not necessarily evidence of absence. But, it has to be realized that much of the most convincing evidence for the role of lipid peroxidation in vivo, in the final analysis, is mostly indirect evidence and that many of our conclusions have been drawn by inference.

Nevertheless, the concept of lipid peroxidation is one of the truly important concepts of current experimental pathology and toxicology. It has created discussion, stimulated the planning of careful and ingenious experiments, and given us many sound data. To us, it appears to be a hypothesis that remains, and deserves, to be tested extensively.

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